JC10 Rec'd PCT/PTO 18 DEC 2001

FORM PTO-1390 (REV. 9-2001) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY 'S DOCKET NUMBER: REG 720-US

U S APPLICATION NO. (If known, see 37 CFR 1.5): NOT YET KNOWN 10/018761

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US00/17173	June 22, 2000	July 7, 1999

TITLE OF INVENTION: METHODS OF INHIBITING MUSCLE ATROPHY

APPLICANT(S) FOR DO/EO/US: David J. Glass, Christian Rommel, and George D. Yancopoulos

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. [ ] The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau
   c. [X] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [X] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. [ ] is attached hereto.
   b. [ ] has been previously submitted under 35 U.S.C. 154(d)(4).
7. [X] Amendments to the claims of the International Aplication under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371 (c)(3))
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English lanugage translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
14. [ ] A SECOND or SUBSEQUENT preliminary amendment.
15. [ ] A substitute specification.
16. [ ] A change of power of attorney and/or address letter.
17. [ ] A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. [ ] A second copy of the published international application under 35 U S.C. 154(d)(4).
19. [ ] A second copy of the English language translation of the international application under 35 U.S C. 154(d)(4).
20. [X] Other items or information: an unexecuted Declaration and Power of Attorney, an Associate Power of Attorney, and bearing Express Mail Label No. ET712522462US dated December 18, 2001.

ET 712522462 US

531 Rec'd PC. 18 DEC 2001

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
NOT YET KNOWN 10/018761	PCT/US00/17173	REG 720-US

21. [X] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$890.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 740.	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	$	
Total claims	41 - 20 =	21	x **$18.00**	$ 378.	
Independent claims	6 - 3 =	3	x **$84.00**	$ 252.	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$ 0.	
TOTAL OF ABOVE CALCULATIONS =				$ 1,370.	
[ ] Applicant claims small entity status See 37 CFR 1.27. The fees indicated above are reduced by 1/2. +				$	
SUBTOTAL =				$ 1,370.	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 1,370.	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 1,370.	
				Amount to be refunded:	$
				charged:	$

a. [ ] A check in the amount of $ _____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 18-0650 in the amount of $ 1,370. to cover the above fees. A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 18-0650. A duplicate copy of this sheet is enclosed.

d. [ ] Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137 (a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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777 Old Saw Mill River Road
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Laura Fischer
SIGNATURE

Laura Fischer
NAME

P-50,420
REGISTRATION NUMBER

10/018761
531 Rec'd PCT/PTO 18 DEC 2001

Att. Docket No. REG 720-US

IN THE UNITED STATES DESIGNATED OFFICE/ELECTED OFFICE

In re Application Of	:	David J. Glass, Christian Rommel, and George D. Yancopoulos
Int'l File No.	:	PCT/US00/17173
Int'l File Date	:	June 22, 2000
USSN	:	Not Yet Known
Filed	:	Filed Herewith
For	:	

December 18, 2001

Commissioner for Patents
U.S. Patent and Trademark Office
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

This paper is submitted in connection with the above-identified application. Prior to examination of the application on the merits, please amend the specification as follows:

In the Specification:

Please replace the paragraph starting on page 1, line 3, with the following:

This application claims priority of International Patent Application PCT/US00/17173, filed June 22, 2000, which claims priority of U.S. Provisional Application No. 60/142,857, filed July 7, 1999, the entirety of which is incorporated by reference herein.

10/018761
MARKED-UP VERSION 531 Rec'd PCT/P 18 DEC 2001

METHODS OF INHIBITING MUSCLE ATROPHY

This application claims priority of International Patent Application NO. PCT/US00/17173 filed June 22, 2000, which
~~This application~~ claims priority of U.S. Provisional Application No. 60/142,857, filed July 7, 1999, the entirety of which is incorporated by reference herein.

BACKGROUND OF THE INVENTION

A decrease in muscle mass, or atrophy, is associated with various physiological and pathological states. For example, muscle atrophy can result from denervation due to nerve trauma; degenerative, metabolic or inflammatory neuropathy, e.g. Guillian-Barré syndrome; peripheral neuropathy; or nerve damage caused by environmental toxins or drugs. Muscle atrophy may also result from denervation due to a motor neuropathy including, for example, adult motor neuron disease, such as Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease); infantile and juvenile spinal muscular atrophies; and autoimmune motor neuropathy with multifocal conductor block. Muscle atrophy may also result from chronic disease resulting from, for example, paralysis due to stroke or spinal cord injury; skeletal immobilization due to trauma, such as, for example, fracture, ligament or tendon injury, sprain or dislocation; or prolonged bed rest. Metabolic stress or nutritional insufficiency, which may also result in muscle atrophy, include *inter alia* the cachexia of cancer and other chronic illnesses including AIDS, fasting or rhabdomyolysis, and endocrine disorders such as disorders of the thyroid gland and diabetes. Muscle atrophy may also be due to a muscular dystrophy syndrome such as Duchenne, Becker, myotonic, fascioscapulohumeral, Emery-Dreifuss, oculopharyngeal, scapulohumeral, limb girdle, and congenital types, as well as the

Att. Docket No. REG 720-US
Int'l File No.: PCT/US00/17173
Int'l File Date: June 22, 2000
USSN: Not Yet Known
US File Date: Filed Herewith
Preliminary Amendment

REMARKS

This Preliminary Amendment is made merely to add the priority date to the application. Applicants submit herewith as Exhibit A: Marked-Up Version of amended page 1 of the specification.

No fee is deemed necessary for filing this paper. However, if any fees are deemed necessary, the Commissioner is hereby authorized to charge any such fees required by this paper to Deposit Account No. 18-0650.

Respectfully submitted,

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10/018761

METHODS OF INHIBITING MUSCLE ATROPHY

This application claims priority of U.S. Provisional Application No. 60/142,857, filed July 7, 1999, the entirety of which is incorporated by reference herein.

BACKGROUND OF THE INVENTION

A decrease in muscle mass, or atrophy, is associated with various physiological and pathological states. For example, muscle atrophy can result from denervation due to nerve trauma; degenerative, metabolic or inflammatory neuropathy, e.g. Guillian-Barré syndrome; peripheral neuropathy; or nerve damage caused by environmental toxins or drugs. Muscle atrophy may also result from denervation due to a motor neuropathy including, for example, adult motor neuron disease, such as Amyotrophic Lateral Sclerosis (ALS or Lou Gehrig's disease); infantile and juvenile spinal muscular atrophies; and autoimmune motor neuropathy with multifocal conductor block. Muscle atrophy may also result from chronic disease resulting from, for example, paralysis due to stroke or spinal cord injury; skeletal immobilization due to trauma, such as, for example, fracture, ligament or tendon injury, sprain or dislocation; or prolonged bed rest. Metabolic stress or nutritional insufficiency, which may also result in muscle atrophy, include *inter alia* the cachexia of cancer and other chronic illnesses including AIDS, fasting or rhabdomyolysis, and endocrine disorders such as disorders of the thyroid gland and diabetes. Muscle atrophy may also be due to a muscular dystrophy syndrome such as Duchenne, Becker, myotonic, fascioscapulohumeral, Emery-Dreifuss, oculopharyngeal, scapulohumeral, limb girdle, and congenital types, as well as the

dystrophy known as Hereditary Distal Myopathy. Muscle atrophy may also be due to a congenital myopathy, such as benign congenital hypotonia, central core disease, nemalene myopathy, and myotubular (centronuclear) myopathy. Muscle atrophy also occurs during the aging process.

Muscle atrophy in various pathological states is associated with enhanced proteolysis and decreased production of muscle proteins. Muscle cells contain lysosomal proteases and cytosolic proteases. The cytosolic proteases include Ca^{2+}-activated neutral proteases (calpains) and an ATP-dependent ubiquitin-proteasome proteolytic system. The lysosomal and cytosolic systems are capable of degrading muscle proteins in vitro, but less is known about their roles in proteolysis of muscle proteins in vivo. Some studies have reported that proteasome inhibitors reduce proteolysis in atrophying rat skeletal muscle (e.g. Tawa et al. (1997) J. Clin. Invest. 100:197), leading to suggestions that the ubiquitin-proteasome pathway has a role in the enhanced proteolysis. However, the precise mechanisms of proteolysis in atrophying muscle remain poorly characterized.

Ras is a signaling molecule, found in every cell in the body. It is activated by a variety of growth factors, including growth factors which signal tyrosine kinase receptors. Ras activation results in activation of several downstream signaling pathways, including the Raf/Mek/Erk pathway. Other potential downstream substrates of Ras include the following signaling molecules: PKC, Ral-GDS, KSR, and Rin-1 (Trends Genet 1999 Apr;15(4):145-9). Ras, Raf, Mek and Erk genes are well-conserved through evolution, and homologs can be found in eukaryotes from yeast to humans (Ras: Taparowsky E, et al. (1982)

Nature 300:762-5; Raf: Heidecker, et al. (1990) Mol. Cell. Biol. 10:2503; Samuels, et al. (1993) Mol. Cell. Biol. 13:6241; Morrison, et al. (1997) Curr. Opin. Cell Biol. 9:174; Mek: Crews CM, et al. (1992) Science 258:478-80; Erks: Boulton TG, et al. (1991) Cell 65 :663-75).

When a constitutively active mutant of Ras was expressed in cardiac muscle in transgenic mice, the animals were born with hypertrophic heart tissue (J Biol Chem. 1995 Sep 29;270(39):23173-8). This suggested the possibility that Ras activation might lead to hypertrophy in skeletal muscle as well, and indicated that Ras activation might be an efficacious method to reverse skeletal muscle atrophy.

However, it has been discovered in accordance with the present invention that proteolysis or decreased synthesis in muscle cells is actually increased by activation of cell signaling molecules in the Ras and downstream Raf/Mek/Erk/ pathways.

SUMMARY OF THE INVENTION

The present invention provides a method of inhibiting skeletal muscle atrophy, by inhibiting signaling pathways that lead to skeletal muscle atrophy. Agents that inhibit Ras activation, or which inhibit signaling molecules downstream of Ras, such as Raf/Mek/Erk, in the present method are useful for preventing or reducing atrophy and/or causing hypertrophy in skeletal muscle cells.

the transgene, injection of the transfected ES cells into host blastocysts into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mice. The constitutively active mutant form of Ras, Raf, Mek, or Erk nucleic acid is expressed in muscle cells of the transgenic mice to provide the constitutively active mutant form of the Ras, Raf, Mek or Erk protein in the muscle cells.

In another preferred embodiment, the transgenic mouse is made by injection of a vector comprising the transgene directly into the pronucleus of a fertilized egg, as described in Hogan et al., supra. The injected egg is inserted into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mouse.

The presence of constitutively active mutant forms of Ras, Raf, Mek or Erk protein can also be assessed using antibodies specific for these proteins, or by antibodies specific for an epitope-tag which is genetically inserted into the vectors encoding these proteins.

In methods for the identification of an agent that inhibits muscle cell atrophy, the agent may be contacted with the muscle cell expressing constitutively active mutant forms of Ras, Raf, Mek or Erk by methods known in the art. For cells in culture or cells obtained from transgenic organisms, the cell may be contacted with the agent by, for example, direct application. The agent may be modified or contained in a delivery vehicle to facilitate entry into the cell. The agent may be isolated and purified, or it may present in a sample or composition to be subjected to further purification subsequent to a positive result in the present method. For example, the agent may be contained in a cell

It has been discovered in accordance with the present invention that when skeletal muscle cells are treated with a chemical which blocks signaling of the Mek kinase, which is downstream of Ras and Raf, the resultant cells appear hypertrophic in comparison to control cells.

Accordingly, Applicants have discovered that inhibitors of Ras or the downstream signaling molecules Raf/Mek/Erk are useful for reducing or preventing atrophy in skeletal muscle cells. Further, such inhibitors may be used to decrease and/or prevent atrophy in mammals having a condition, such as those described herein, in which skeletal muscle atrophy is occurring. In a preferred embodiment, such inhibitors are those already proven to be pharmacological antagonists for Ras [Cancer Chemother Pharmacol. (1999) 43(1):50-8], or Raf/Mek/Erk, such as PD98059 [Proc. Natl. Acad. Sci. (1995) 92:7686] or farnesyl transferase (Chem. Biol. (1995) Dec 2(12):787-91). According to this embodiment, atrophying skeletal muscle cells, or vertebrate animals having a condition as described above in which muscle cells are atrophying, are treated with an inhibitor so as to prevent or decrease muscle cell atrophy. Such treatment may be utilized prophylactically prior to the onset of muscle atrophy or after such condition has manifested itself. Vertebrate animals include any species containing skeletal muscle and a backbone, and includes chickens, rodents, rabbits, dogs, cats, cows, horses, pigs, sheep, primates and humans, preferably humans.

In another embodiment, inhibitors of Ras or the downstream signaling molecules Raf/Mek/Erk may be used to cause hypertrophy in skeletal muscle cells. Further, such inhibitors may be used to cause muscle hypertrophy in vertebrate animals having conditions, such as those described herein, in which skeletal muscle atrophy is anticipated. In

some settings, such as in animals farmed for meat production, such agents might be used to increase meat production. In a preferred embodiment, such inhibitors are those already proven to be pharmacological antagonists for Ras [Cancer Chemother Pharmacol. (1999) 43(1):50-8], or Raf/Mek/Erk, such as PD98059 [Proc.Natl. Acad. Sci. (1995) 92:7686] or farnesyl transferase transferase (Chem. Biol. (1995) Dec 2(12):787-91). According to this embodiment, atrophying skeletal muscle cells, or vertebrate animals having a condition as described above in which muscle cells are atrophying, are treated with an inhibitor so as to prevent or decrease muscle cell atrophy. Such treatment may be utilized prophylactically prior to the onset of muscle atrophy or after such condition has manifested itself. Alternatively, such treatment may be used to induce skeletal muscle hypertrophy.

The invention further provides for therapeutic compositions comprising a Ras or Raf/Mek/Erk antagonist in a carrier which may include excipients, diluents or other pharmaceutically active compounds. Such compositions may be administered systemically or locally. Any appropriate mode of administration known in the art may be used, including, but not limited to, intravenous, intrathecal, intraarterial, intranasal, oral, subcutaneous, intraperitoneal, or by local injection or surgical implant. Sustained release formulations are also provided for.

The activity of the compositions of the invention in vertebrate animals may be assessed using experimental animal models of disorders in which muscle atrophy is present. For example, the activity of the compositions may be tested for their effect in the hindlimb immobilization model described herein in Example 2. Alternatively, the activity of the compositions may be assessed in animals using

experimental animals in which hypertrophy can be measured. For example, the activity of the compositions may be tested for their effect on muscles undergoing exercise-induced hypertrophy, or compensation-induced hypertrophy. Alternatively, the muscle may be assessed in control animals as compared to animals treated with the experimental compositions, to determine if the treated animals exhibit skeletal muscles hypertrophy as a result of their treatment. Data obtained from cell culture assays and animal studies can be used in formulating a range of dosages for use in vertebrate animals, including humans. The dosage of the compositions of the invention should lie within a range of serum circulating concentrations with little or no toxicity. The dosage may vary within this range depending on the dosage form employed and the route of administration.

In another embodiment, the present invention provides a method of identifying an agent that inhibits atrophy or causes hypertrophy in muscle cells, comprising creating muscle cells that express constitutively active mutants of Ras or Raf/Mek/Erk, contacting said muscle cells with an agent to be tested; and screening for cells which do not undergo atrophy or in which the amount of atrophy is reduced as compared to control cells.

In one embodiment, the present invention provides a method of identifying an agent that inhibits atrophy or causes hypertrophy in muscle cells, comprising preparing an *in vitro* assay for Ras or Raf/Mek/Erk activity, contacting the Ras or Raf/Mek/Erk with an agent to be tested; and screening for agents which block the Ras or Raf/Mek/Erk activity.

Another preferred embodiment of the present invention provides a method of identifying a gene encoding a gene-product that inhibits atrophy or causes hypertrophy in muscle cells, comprising preparing muscle cells expressing constitutively active mutants of Ras or Raf/Mek/Erk; introducing into these muscle cells a gene to be tested, under conditions in which said test gene encodes a product, measuring the amount of atrophy in said test-gene encoding muscle cells and comparing the amount of atrophy in said test-gene encoding cells with the amount of atrophy in the muscle cells in which the test gene has not been introduced, wherein a smaller amount of atrophy in the test-gene encoding muscle cells indicates that the test gene product inhibits the Ras or Raf/Mek/Erk pathway and therefore inhibits atrophy in cells under conditions in which such atrophy is occurring.

Cells useful for expressing constitutively active mutant forms of Ras or Raf/Mek/Erk include any and all muscle cells that can be maintained in culture and that can be engineered to express a heterologous nucleic acid. The cells may be primary cultures or established cell lines. Suitable muscle cells include myoblasts, for example the C2C12 cell line as described in Bains, et al. (1984) Mol. Cell. Biol. 4:1449-1553, the disclosure of which is incorporated herein by reference. Other suitable muscle cells include Sol8 cells, described by Glass et al. (1997) Proc. Natl. Acad. Sci. USA 16:8848, and L6 cells, described by Ringentz et al. (1978) Exp. Cell Res. 113:233, the disclosures of which are incorporated herein by reference.

Cells useful for making cultured muscle cells expressing the Ras or Raf/Mek/Erk genes also include non-muscle cells which may be differentiated into skeletal muscle cells; an example of this type of

cell is an embryonic stem-cell, which can be differentiated into a skeletal muscle cell by insertion of the muscle-specific transcription factor, MyoD, as described by Shani et al. (1992) Symp. Soc. Exp. Biol. 46:19. Another example of a non-muscle cell which can be differentiated along a skeletal muscle pathway is a rhabdomyosarcoma tumor cell, which can be differentiated by contact with retinoic acid, as described by Gabbert et al. (1988) Cancer Res. 48:5264.

The constitutively active mutant forms of the Ras or Raf/Mek/Erk nucleic acids under the control of suitable transcriptional and translational regulatory sequences can be introduced into the cell by methods known in the art including, for example, transformation, transfection, infection, transduction and injection. In a preferred embodiment, the Ras, Raf, Mek or Erk gene is contained in an expression vector under the control of a suitable promoter to effect expression in muscle cells. Preferred promoters include skeletal actin, such as human skeletal actin (HSA), chicken skeletal actin (CSA), mouse skeletal actin (MSA), rat skeletal actin (RSA); muscle creatine kinase (MCK); MyoD; MRF4; myogenin; dystrophin; utrophin; MuSK; myosin light chain (MLC1/3); myosin heavy chain (MHC). Other non-muscle specific promoters include cytomegalovirus (CMV); LTR (long-terminal repeat, found in retroviruses including the Moloney Murine Leukemia Virus - MuLV); HTLV-1 (the promoter for Human T-cell leukemia virus 1); late, middle, or early Adenovirus.

The amount of atrophy in cells may be measured by quantitation of diameter, protein amount, or by activation of the p70S6 kinase or Phas-1 protein, which stimulates protein synthesis. In transgenic organisms, muscle atrophy may be measured as described in Example 2 herein.

In yet another preferred embodiment, Ras, Raf, Mek or Erk protein is expressed in an *in vitro* assay, such that agents can be screened which inhibit these signaling molecules. Alternatively, *in vitro* binding assays may be utilized to identify agents which block the binding of Ras, Raf, Mek or Erk to downstream signaling components, using, for example, technology as described in End, et al. (1993) J. biol. Chem. 268(14):10066-10075.

The expression vector containing the constitutively active mutant form of the Ras, Raf, Mek or Erk nucleic acid under the control of a suitable promoter is introduced into the cell by known methods, for example liposome-mediated transfection, calcium phosphate-mediated transfection, DEAE-dextran transfection, naked DNA transfection, microinjection, electroporation, retroviral-mediated infection, adenoviral-mediated infection, or adeno-associated viral-mediated infection. The constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid can be introduced into the cell stably or transiently. Methods for introducing heterologous nucleic acids into eukaryotic cells are described in numerous laboratory manuals including, for example, DNA Cloning: A Practical Approach, vols. I-III (1985) Glover, ed., IRL Press Limited, Oxford, and Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor, NY.

In a preferred embodiment, the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid is inserted into a retroviral vector, for example, as described by Pear et al. (1993) Proc. Natl. Acad. Sci. USA 90:8392, incorporated herein by reference. In this embodiment, the viral LTR promoter controls the transcription of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid. The vector is transiently transfected into a retroviral packaging line, and the resulting recombinant virus which contains the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid is harvested, as described by Pear et al., id. The recombinant virus is then used to infect myoblasts as described by Hoffman et al. (1996) Proc. Natl. Acad. Sci. USA 93:5185, incorporated herein by reference. C2C12 cells, as an example of skeletal muscle cells, expressing constitutively active mutant forms of Ras, Raf, Mek, or Erk, can be maintained in the undifferentiated state by growing them in tissue culture media containing at least 10% fetal calf serum, or they can be differentiated into skeletal muscle myotubes by growing them in media containing 2% horse serum. The necessary tissue culture methods are known to those of ordinary skill in the art. C2C12 cells are described in Bains et al. (1984) Mol. Cell Biol. 4:1449, incorporated herein by reference.

Muscle cells that express the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid may be contained within, or obtained from, a transgenic organism. The term transgenic organism as used herein includes muscle cell-containing multicellular organisms that have a foreign gene, and in particular the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid, incorporated into their genome. Transgenic organisms contemplated in accordance with the

present invention include worms, birds, chickens, turkeys, flies and non-human mammals such as, for example, mice, rats, dogs, rabbits, sheep, pigs, sheep, goats, horses and cattle. Transgenic worms and mice are particularly preferred for research use in accordance with the present invention.

In the transgenic organisms of the present invention, the coding region of the constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid under the control of a muscle-specific regulatory element is integrated into the genome of the host organism. The muscle-specific regulatory element directs expression of constitutively active mutant forms of the Ras, Raf, Mek, or Erk nucleic acid to provide expression of these nucleic acids in muscle cells of the host organism, and may be native to the host organism, or heterologous to the host organism. Preferred muscle-specific regulatory elements useful for the present invention are selected for optimal expression in the particular host. Preferred muscle-specific regulatory elements for use in non-human transgenic mammals include the human skeletal actin (HSA) promoter (Brennan et al. (1993) J. Biol. Chem. 268:719, incorporated herein by reference) and the muscle creatine kinase (MCK) promoter (Johnson et al. (1989) Mol. Cell. Biol. 9:3393, incorporated herein by reference).

The transgenic organisms of the present invention are made by transferring a transgene DNA construct comprising the coding region of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid operably linked to a muscle-specific promoter into a host organism such that the transgene is integrated into the genome of the host organism. Methods for making transgenic non-human organisms

are known in the art and include, for example, DNA microinjection, embryonic stem (ES) cell transfer, retroviral infection, blastomere-embryo aggregation, teratocarcinoma cell transfer, electrofusion, nuclear transplantation, and spermatozoa-mediated transfer. The methods are reviewed, for example by Pinkert et al., "Transgenic Animal Modeling" in Molecular Biology and Biotechnology, Meyers, ed., VCH Publishers, Inc., New York, 1995, pp. 90-907 and numerous laboratory manuals including, for example, Hogan et al. (1994) Manipulating the Mouse Embryo: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Plainview, New York, the disclosures of which are incorporated herein by reference. In a preferred embodiment of the present invention, the transgenic non-human organisms are produced by microinjection. A transgenic organism contemplated in accordance with the present invention includes, but is not limited to, a transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse. In a preferred embodiment of the present invention, the transgenic organism is a transgenic mouse. Methods for making transgenic worms, in particular Caenorhabditis elegans, are also known in the art and disclosed, for example by Mello et al. (1991) EMBO. J. 10:3959.

In a preferred embodiment of the present invention, the transgenic organism is a transgenic mouse whose germ cells and somatic cells contain a transgene comprising the coding region of the constitutively active mutant form of the Ras, Raf, Mek, or Erk nucleic acid operably linked to a muscle-specific promoter, whereby the constitutively active mutant form of Ras, Raf, Mek, or Erk protein is thus produced in the muscle cells of the transgenic organism. The transgenic mouse is preferably made by transfection of ES cells with a vector comprising

the transgene, injection of the transfected ES cells into host blastocysts into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mice. The constitutively active mutant form of Ras, Raf, Mek, or Erk nucleic acid is expressed in muscle cells of the transgenic mice to provide the constitutively active mutant form of the Ras, Raf, Mek or Erk protein in the muscle cells.

In another preferred embodiment, the transgenic mouse is made by injection of a vector comprising the transgene directly into the pronucleus of a fertilized egg, as described in Hogan et al., supra. The injected egg is inserted into pseudopregnant foster mother hosts, followed by embryonic development and birth of the transgenic mouse.

The presence of constitutively active mutant forms of Ras, Raf, Mek or Erk protein can also be assessed using antibodies specific for these proteins, or by antibodies specific for an epitope-tag which is genetically inserted into the vectors encoding these proteins.

In methods for the identification of an agent that inhibits muscle cell atrophy, the agent may be contacted with the muscle cell expressing constitutively active mutant forms of Ras, Raf, Mek or Erk by methods known in the art. For cells in culture or cells obtained from transgenic organisms, the cell may be contacted with the agent by, for example, direct application. The agent may be modified or contained in a delivery vehicle to facilitate entry into the cell. The agent may be isolated and purified, or it may present in a sample or composition to be subjected to further purification subsequent to a positive result in the present method. For example, the agent may be contained in a cell

lysate, conditioned cell culture media, or a library of synthetic or naturally occurring compounds. For muscle cells present in a transgenic organism, the cells may be contacted with the agent by delivering the agent by methods known in the art, for example by ingestion, parenteral administration, or direct application to tissue surfaces, and may be present in a composition comprising a carrier or diluent. Agents that may be tested in the method of the present invention include, for example, organic and inorganic molecules such as proteins, peptides, lipids, carbohydrates, nucleic acids, including antisense, metals, salts and so on.

In methods for identifying a gene, the gene product of which inhibits atrophy in muscle cells in the presence of constitutively active mutant forms of Ras, Raf, Mek or Erk, the gene to be tested may be introduced into the muscle cell by methods known in the art and described hereinabove for inhibition of the Ras, Raf, Mek or Erk signaling effects. The gene is under the control of a promoter, as described hereinabove, and may be present in an expression vector. Pools of genes, for example cDNA libraries, may be tested for their ability to interfere with proteolysis. Upon identification of a gene pool that inhibits proteolysis, the pool may be progressively divided and tested until a single gene is identified.

In methods for identifying a gene, the expression of which inhibits atrophy in muscle cells containing constitutively active mutant forms of Ras, Raf, Mek or Erk, genetic mutations may be introduced into a gene in muscle cells by methods known in the art. Methods of directed mutagenesis are well-known in the art, and may be found in laboratory manuals including for example, Directed Mutagenesis: A Practical

Approach (1991) McPherson, ed., Oxford University Press, Oxford. In a preferred embodiment, random mutagenesis is accomplished using the chemical EMS, which can be accomplished by those skilled in the art. An example can be found in Chanal, et al., (1997) Genetics 146:20. Methods to induce mutations are reviewed by Anderson (1995) Methods Cell Biol. 48:31.

The methods of the present invention are useful for the identification of agents, genes and gene products that interfere with atrophy in muscle cells containing constitutively active mutant forms of Ras, Raf, Mek or Erk. The agents, genes and gene products identified by the present methods are useful for the treatment and prevention of muscle atrophy.

References cited hereinabove are incorporated herein in their entirety.

The following non-limiting examples serve to further illustrate the present invention.

EXAMPLES

Example 1

The Effect of Activation and Blocking of Raf in Muscle Cells

To test the effects of activating and blocking Raf in muscle cells, a strain of myoblasts called C2C12 cells were transfected with DNA expression vectors containing either a constitutively active Raf gene [Heidecker, et al. (1990) Mol. Cell. Biol. 10:2503; Samuels, et al. (1993) Mol. Cell. Biol. 13:6241; Morrison, et al. (1997) Curr. Opin. Cell Biol.

9:174] or a dominant negative Raf gene [Bruder, et al. (1992) Genes Dev. 6:545], or, as a control, the expression vector without any gene inserted. A "dominant negative" construct is able to block activity of the endogenous gene product; a "constitutively active" construct encodes a mutated form of the gene which is always in the activated state. These constructs were made using standard molecular biology techniques, and were then stably transfected into C2C12 cells, using the calcium phosphate transfection method. Transfected cells were selected by antibiotic selection. The resulting myoblasts were then grown to confluence, and differentiated into "myotubes", which are multi-nucleated muscle cells, similar to actual muscle fibers.

Myotubes expressing the dominant negative form of Raf were found to be much larger than control myotubes - the myotubes were longer, and had broader diameters. Analysis of Phas-1, a molecule involved in protein synthesis [Xu, et al. (1998) J. Biol. Chem. 273(8):4485-4491] demonstrated that inhibition of Raf causes an increase in Phas-1 activation, thus demonstrating that blocking Raf increases muscle protein synthesis.

Myotubes expressing the constitutively active form of Raf were found to be significantly thinner than control myotubes. Analysis of Phas-1 in these myotubes demonstrated that activation of Raf causes a decrease in Phas-1 activation, demonstrating that activation of Raf decreases muscle protein mass, and that inhibition of Raf may be used to decrease or prevent muscle atrophy.

In addition, C2C12 myotubes were treated with the Mek 1/2 inhibitor known as PD98059 [Dudley, et al. (1995) Proc. Natl. Acad. Sci., 92:

7686], and compared to differentiated cells expressing dominant negative Raf. Cells treated one day post-differentiation with 3 mM of the Mek-inhibiting drug were indistinguishable from dominant negative Raf cells.

Example 2

Animal Model for Testing for Muscle Atrophy

To test for muscle atrophy, the ankle joint of rodents (mice or rats) are immobilized at 90 degrees of flexion. This procedure induces atrophy of the muscles with action at the ankle joint (e.g. soleus, medial and lateral gastrocnemius, tibialis anterior) to varying degrees. A reproducible amount of atrophy can be measured in hindlimb muscles over a 14-day period.

The immobilization procedure may involve either casting (mice) or pinning the ankle joint (rats). Rodents are anesthetized with ketamine/xylazine and the right ankle joint is immobilized. In rats, a 0.5 cm incision is made along the axis of the foot, over the heel region. A threaded screw (1.2 x 8mm) is then inserted through the calcaneous and talis, into the shaft of the tibia. The wound is closed with skin glue. In mice, the ankle joint is fixed at 90 degrees with a light weight casting material (VET-LITE) around the joint. The material is soaked in water and then wrapped around the limb. When the material dries it is hard, but light in weight.

At seven and 14 days following the immobilization, animals are anesthetized and killed by cervical dislocation. The tibialis anterior (TA), medial gastrocnemius (MG), and soleus (Sol) muscles are removed

from the right (immobilized) and left (intact) hindlimbs, weighed, and frozen at a fixed length in liquid nitrogen cooled isopentane. A cohort of control animals which are the same weight and age as the experimental animals are also killed and the muscles removed, weighed and frozen. The amount of atrophy is assessed by comparing the weight of the muscles from the immobilized limb with the weight of the muscles from the control animals.

Example 3

Preparation of Muscle-Specific Eukaryotic Expression Constructs for Constitutively Active Ras and Constitutively Active Raf

DNA encoding constitutively active Ras and DNA encoding constitutively active Raf, was cloned into eukaryotic expression vectors by standard molecular biology techniques. Briefly the DNA encoding a constitutively active RasV12, and DNA encoding the COOH terminal of Raf (RafCT), which is constitutively active when expressed, was gel-purified and cloned into eukaryotic expression vectors containing the Muscle Creatine Kinase (MCK) promoter. These vectors were called MCK RasV12 or MCK RafCT, respectively. MCK promoters have been previously shown to drive muscle-specific expression of transgenes in mice [Johnson et al. (1989) Mol. Cell. Biol. 9:3393].

Example 4

Transfecting C2C12 Cells

Murine C2C12 myoblasts were used as a means of studying the transgenes in cell culture. To achieve Ras and Raf gene expression, vectors using the murine muscle creatine kinase (MCK) gene promoter

as described in Example 3 were transfected into C2C12 cells by calcium phosphate precipitation. To allow for the positive selection of transgenic C2C12 cells, using the drug G418 (Gibco), the MCK- RasV12, and RafCt vectors were co-transfected with a plasmid containing a LTR gene promoter driving a *neo* gene. Expression of this plasmid allows a cell to resist the killing effect of the drug G418; thus, only cells which have been successfully transfected will survive when treated with G418.

The transfected C2C12 cell colonies were harvested, trypsinized, and re-plated for growth in individual wells of a 24-well culture plate. Control C2C12 cells, C2C12 cells containing the MCK RasV12 construct and C2C12 cells containing the MCK RafCT construct were grown to confluence. The serum level was dropped to allow fusion of the myoblasts into differentiated muscle cells. C2C12 cells differentiate into multi-nucleated myotubes. Phenotypes of resultant myotubes were quantitated by diameter, protein amount, and by activation of the Phas-1 protein, which stimulates protein synthesis.

WE CLAIM:

1. A method of inhibiting atrophy in skeletal muscle cells comprising treating the cells with an inhibitor of the Ras/Raf/Mek/Erk pathway.

2. The method of Claim 1 wherein the inhibitor inhibits Ras.

3. The method of Claim 1 wherein the inhibitor inhibits Raf.

4. The method of Claim 1 wherein the inhibitor inhibits Mek.

5. The method of Claim 1 wherein the inhibitor inhibits Erk.

6. The method of Claim 1 wherein the inhibitor is PD98059 or farnesyl transferase.

7. A method of identifying an agent that inhibits atrophy in skeletal muscle cells comprising:

(a) preparing muscle cells that express constitutively active mutant forms Ras/Raf/Mek/Erk;

(b) subjecting the cells to a test agent;

(c) measuring the amount of atrophy in the muscle cells subjected to a test agent;

(d) comparing the amount of atrophy in the muscle cells subjected to a test agent with the amount of atrophy in untreated transgenic muscle cells of step (a), wherein a smaller amount of atrophy in the muscle cells subjected to a test agent indicates that the agent inhibits the Ras/Raf/Mek/Erk pathway and therefore inhibits atrophy in muscle cells.

8. The method of Claim 7 wherein the measuring utilizes muscle cell diameter, protein amount, p70S6 kinase activation or Phas-1 activation.

9. The method of Claim 7 wherein the measuring utilizes measuring inhibition of Ras/Raf/Mek/Erk.

10. The method of Claim 7 wherein the muscle cells are cultured cells.

11. The method of Claim 10 wherein the cultured cells are myoblasts.

12. The method of Claim 11 wherein the myoblasts are C2C12 cells.

13. The method of Claim 11 wherein the myoblasts are differentiated myoblasts.

14. The method of Claim 13 wherein the differentiated myoblasts are myotubes.

15. The method of Claim 7 wherein the muscle cells are obtained from a transgenic organism.

16. The method of Claim 7 wherein the muscle cells are within a transgenic organism.

17. The method of Claim 15 wherein the transgenic organism is a transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse.

18. The method of Claim 16 wherein the transgenic organism is a transgenic transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse.

19. A method of identifying an agent that inhibits atrophy in muscle cells comprising:

a) measuring the activation of the Ras/Raf/Mek/Erk pathway in untreated muscle cells,

b) subjecting the muscle cells that express the Ras/Raf/Mek/Erk pathway to a test agent,

c) measuring the amount of Ras/Raf/Mek/Erk activity in the muscle cells subjected to a test agent;

d) comparing the amount of Ras/Raf/Mek/Erk activity in the muscle cells subjected to a test agent with the amount in the untreated muscle cells, whereby a larger amount in the muscle cells treated with a test agent indicates that the agent inhibits the Ras/Raf/Mek/Erk pathway and therefore inhibits atrophy in muscle cells.

20. A method of identifying a gene encoding a gene product that inhibits skeletal muscle atrophy comprising:

(a) preparing muscle cells that express constitutively active mutant forms Ras/Raf/Mek/Erk;

(b) introducing into the cells of (a) a test gene under conditions in which the test gene encodes a product;

(c) measuring the amount of atrophy in the test-gene encoding muscle cells; and

(d) comparing the amount of atrophy in the test-gene encoding cells with the amount of atrophy in the muscle cells of step (a) in which the test gene has not been introduced, wherein a smaller

amount of atrophy in the test gene-encoding muscle cells indicates that the test gene product inhibits the Ras/Raf/Mek/Erk pathway and therefore inhibits atrophy in muscle cells.

21. The method of Claim 20 wherein the measuring utilizes muscle cell diameter, protein amount, p70S6 kinase activation or Phas-1 activation.

22. The method of Claim 20 wherein the muscle cells are cultured cells.

23. The method of Claim 22 wherein the cultured cells are myoblasts.

24. The method of Claim 23 wherein the myoblasts are differentiated myoblasts.

25. The method of Claim 20 wherein the muscle cells are obtained from a transgenic organism.

26. The method of Claim 20 wherein the muscle cells are within a transgenic organism.

27. The method of Claim 25 wherein the transgenic organism is a transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse.

28. The method of Claim 26 wherein the transgenic organism is a transgenic fly, worm, bird, chicken, turkey, mouse, rat, dog, cat, rabbit, sheep, pig, goat or horse.

29. A method of inhibiting atrophy in a vertebrate animal having an atrophy-inducing condition comprising treating the vertebrate animal with an effective amount of an inhibitor of Ras, Raf, Mek or Erk.

30. The method of Claim 29 wherein the vertebrate animal is a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate or human.

31. The method of Claim 29 wherein the vertebrate animal is treated prior to exposure to or onset of the atrophy-inducing condition.

32. The method of Claim 29 wherein the atrophy-inducing condition is immobilization.

33. The method of Claim 29 wherein the atrophy-inducing condition is denervation, starvation, nutritional deficiency, metabolic stress, diabetes, aging, muscular dystrophy or myopathy.

34. A method of causing muscle hypertrophy in skeletal muscle cells comprising treating the cells with an inhibitor of the Ras/Raf/Mek/Erk pathway.

35. The method of Claim 34 wherein the inhibitor inhibits Ras.

36. The method of Claim 34 wherein the inhibitor inhibits Raf.

37. The method of Claim 34 wherein the inhibitor inhibits Mek.

38. The method of Claim 34 wherein the inhibitor inhibits Erk.

39. The method of Claim 34 wherein the inhibitor is PD98059 or farnesyl transferase.

40. The method of Claim 34 wherein the muscle cells are within a vertebrate animal.

41. The method of Claim 40 wherein the vertebrate animal is a chicken, rodent, rabbit, dog, cat, cow, horse, pig, sheep, primate or human.

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(54) Title: METHODS OF INHIBITING MUSCLE ATROPHY

(57) Abstract: The present invention provides a method for inhibiting atrophy in muscle cells. It further provides a method for inhibiting skeletal muscle atrophy or causing skeletal muscle hypertrophy in vertebrate animals. It also provides a method of identifying agents, genes and gene products that may be used to reduce proteolysis or increase protein synthesis in muscle cells.

ATT. DOCKET NO. REG 720-US

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled **METHODS OF INHIBITING MUSCLE ATROPHY**, the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

I acknowledge the duty to disclose information of which I am aware that is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

PCT/US00/17173 filed June 22, 2000

I hereby claim the benefit under Title 35, United States Code, §119(E) of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States Application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) that occurred between the filing date of the prior application and the national or PCT international filing date of this application:

USSN 60/142,857 filed July 7, 1999

And I hereby appoint Joseph M. Sorrentino (Registration No. 32,598), Gail M. Kempler (Registration No. 32,143), Laura Fischer (Registration No. P-50,420), and Linda O. Palladino (Registration No. 45,636) each of them my attorneys and agent, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all

business in the Patent and Trademark Office connected therewith and to file any International Applications that are based thereon under the provisions of the Patent Cooperation Treaty.

Please address all communications, and direct all telephone calls, regarding this application to:

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I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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